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ORIGINAL ARTICLE

Effects of glucose, propionate and splanchnic hormones on neuropeptide mRNA concentrations in the ovine hypothalamus

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Summary

The capacity for glucose, propionate or hormones of splanchnic origin to influence appetite by directly regulating the expression of neuropeptides in the feeding centres of the hypothalamus of the ruminant is not described. Therefore, our objective was to measure the direct effect of metabolites (glucose and propionate) or hormones [insulin, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and polypeptide YY (PYY)] on hypothalamic mRNA concentrations for neuropeptide Y (NPY), agouti-related peptide (AgRP) and proopiomelanocortin (POMC) following *in vitro* incubation. Hypothalamic tissue from 4- to 5-month-old lambs was obtained at slaughter and immediately incubated in culture media for 2 h at 36 °C. Treatments included a control Dulbecco's modified Eagle medium (DMEM) containing 1 mM glucose or DMEM with the following additions: 10 mM glucose, 1 mM propionate, 1 nM insulin, 120 pM GLP-1, 100 pM PYY, 80 pM CCK or 10 mM glucose plus 1 nM insulin. The abundance of mRNA for NPY, AgRP and POMC was measured using quantitative reverse transcriptase PCR. Fisher's protected LSD test was used to compare changes in relative mRNA concentrations for the hypothalamus incubated in the control media vs. the rest of the treatments. The media containing glucose plus insulin increased POMC mRNA concentration ($p < 0.05$), but did not affect NPY or AgRP mRNA concentration. There were no effects observed for the other treatments ($p > 0.20$). Results of the present study are consistent with the concept that effects of propionate on feed intake in ruminants is not mediated through direct effects on the hypothalamus, and that insulin is required for an effect of glucose on hypothalamic POMC expression.

Introduction

Despite the importance of dry matter intake (DMI) as a driver of ruminant meat and milk production, much less is known about the mechanisms by which DMI is regulated in ruminants compared with non-ruminants. The role of hypothalamic peptides in the regulation of appetite and feed consumption has been described for a number of non-ruminant species (e.g. Wilding, 2002; Gale et al., 2004). Neuropeptides known to increase appetite include neuropeptide Y (NPY) and agouti-related peptide (AgRP), while proopiomelanocortin (POMC) is a neuropeptide that decreases appetite (Valassi et al., 2008). An increase in hypothalamic concentration of mRNA for NPY and AgRP was observed in fasting compared with *ad libitum*-fed sheep (Adam et al., 2002). However, to our knowledge, there are no

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reports of the direct effects of metabolites or hormones on the hypothalamic concentration of these neuropeptides in ruminants.

In non-ruminants, the synthesis of these hypothalamic peptides is influenced by concentrations of glucose (Lee et al., 2005) and insulin (Schwartz et al., 1992a,b; Gale et al., 2004). In sheep, studies using gold thioglucose (Baile, 1968) and glucose infusions (Manning et al., 1959) found that glucose had little effect on DMI, suggesting that 'glucostatic' regulation of DMI is minimal in ruminants. Ruminants typically absorb a small amount of glucose, and plasma glucose concentrations are relatively constant and maintained primarily by liver synthesis from propionate, which shows little diurnal or postprandial variation. On the other hand, studies in dairy cattle (Sheperd and Combs, 1998; Oba and Allen, 2003) have observed a decrease in DMI during ruminal propionate infusion that was associated with an increase in jugular vein plasma glucose concentration. Under normal conditions, virtually all the propionate absorbed into the portal vein is removed by the liver (Reynolds, 2006), and the effect of propionate on meal size is believed to be a consequence of propionate oxidation and ATP generation in the liver (Oba and Allen, 2003).

In addition to insulin, hormones known to affect feed intake in non-ruminants include cholecystokinin (CCK), glucagon-like peptide-1 (7, 36) amide (GLP-1) and peptide YY_{3-36} (PYY) (Valassi et al., 2008). In ruminants, a decrease in DMI has been associated with an increase in plasma concentration of GLP-1 and CCK (Relling and Reynolds, 2007; Bradford et al., 2008), but to our knowledge, no investigations have reported the direct effect of these gut peptide hormones on gene expression for the hypothalamic neuropeptides. Therefore, our objectives were to test under the conditions of direct *in vitro* incubation whether increased media concentrations of glucose, propionate or hormones (insulin, CCK, GLP-1 and PYY) on mRNA abundance for the orexigenic neuropeptides NPY and AgRP, and the anorexic neuropeptide POMC, in sheep hypothalamic tissue cultured *ex vivo*.

Materials and methods

Hypothalami were removed from 40 market lambs, 5–10 min after slaughter in the abattoir of The Ohio State University Meat Laboratory (Columbus, OH, USA), and used in an incomplete block design experiment. The criterion for blocking was the day of slaughter. The lambs averaged 141 days of age and 54.7 ± 0.3 kg at slaughter. Prior to slaughter, they were fed a diet containing 773 g/kg whole maize grain, 102 g/kg maize grain meal, 97 g/kg soybean meal and 28 g/kg vitamin and mineral supplement. The wethers were euthanized by captive bolt and exsanguination. The top of the skull was removed with a hand saw, and the hypothalamus was removed with a scalpel as described for sheep by Glass et al. (1984). In brief, the frontal land mark for the hypothalamus was the optic chiasm. Caudal of the optic chiasm is the third ventricle. The first incision was a 1.2-cm lateral–lateral cut behind the optic chiasm. Two frontal–caudal cuts of 1.5 cm were made parallel to the third ventricle. The fourth cut closed the rectangular area. A final cut was made at a depth of 0.6 cm to provide a cube-shaped tissue sample with dimensions of 1.2 by 1.5 by 0.6 cm. After the hypothalami were removed, they were further sliced into 2- to 3-mm-thin longitudinal and 5-mm-depth cuts with a sterile scalpel to increase exposure to hormones and metabolites treatments. We assumed that any exposure of hypothalamic cells to concentrations of metabolites and hormones was uniform across blocks. The thin slices of the tissues were incubated in 10 ml of Dulbecco's modified eagle medium (DMEM, #11054; Invitrogen, Carlsbad, CA, USA) containing 1 mM glucose with 1% foetal bovine serum and the hormone or metabolite treatment (described later) for 2 h at 36 °C. After the 2-h incubation, the hypothalami were removed from the media, rinsed with sterile saline solution, flash-frozen in liquid N_2 and stored at −80 °C until RNA was extracted. The 2-h incubation in the experiment was selected based on the responses of mRNA concentrations in a previous study (Lee et al., 2005).

On the first day of sampling (block 1), the treatments ($n = 4$ per treatment) were as follows: control (C), high glucose (HG; 10 mM of glucose), propionate (P; 1 mM of Na propionate), insulin (I; 1 nM of bovine insulin), or glucose–insulin (G + I; 1 nM of insulin and 10 mM of glucose) (Table 1). On the second day of sampling (block 2), the treatments ($n = 4$ per treatment) were as follows: C, HG, CCK [80 pM of CCK-8 sulphide (C2175; Sigma-Aldrich Inc, St Louis, MO, USA)], GLP-1 120 pM of GLP-1 (7-36) amide (H6795; Bachem California Inc, Torrence, CA, USA)] or PYY (100 pM of bovine PYY_{3-36}) (SynPep Corporation, Dublin, CA, USA) (Table 1). Bovine PYY was used because the amino acid sequence of ovine PYY was not known when the experiment was conducted, and we purchased a synthesized PYY_{3-36} because there were no commercial sources of bovine PYY available. The incubation dose used

Table 1 Treatments and number of observations (*n*) used in each day (block) of the experiment

Treatments	Block 1	Block 2
1 mM glucose (C)	4	4
10 mM of glucose (HG)	4	4
1 nM of bovine insulin (I)	4	0
1 mM of Na propionate (P)	4	0
1 nM of insulin and 10 mM of glucose (G + I)	4	0
80 pM of CCK-8 sulphide (CCK)	0	4
120 pM of GLP-1 (7-36) amide (GLP-1)	0	4
100 pM of bovine peptide YY_{3-36} (PYY)	0	4

Table 2 Primer sequences used for the reverse transcriptase quantitative PCR

Item	Forward sequence, 5′ to 3′	Reverse sequence, 5′ to 3′
NPY	TCAGCGCTGCGACACTACAT	GCAGAGACTGGAGAGCAAGT
AgRP	CCTGAGGAAGCCTTATTCCT	CAGGATTCATGCAGCCTTAC
POMC	AGTGTCAGGACCTCACCACG	GCTGCTGCTACCATTCCGA

NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, proopiomelanocortin.

for the HG treatment was chosen because this concentration was shown to increase NPY and AgRP in non-ruminants (Lee et al., 2005). This is approximately three times the normal plasma concentration. For the other treatments, doses were also approximately three times the physiological plasma concentrations reported for ruminants (Onaga et al., 2000; Oba and Allen, 2003; Relling and Reynolds, 2007).

For RNA extraction, the TRIzol® procedure (Invitrogen) was used. Concentration of RNA was determined by measuring absorbance at 260 nm. Reverse transcription (RT)–PCR was performed as described by Ndiaye et al. (2008). The relative mRNA concentration of NPY, AgRP and POMC were determined by RT-quantitative PCR using the DNA Engine Monitor 2 (BioRad Laboratories, Hercules, CA, USA). Primers for NPY, AgRP and POMC were validated in sheep hypothalamic tissue. Oligonucleotide primers for NPY, AgRP and POMC were obtained from Qiagen Operon Biotechnologies (Alameda, CA, USA). The primer sequences used are described in Table 2. Primers were diluted to a working concentration of 15 μM with nuclease-free water (Sigma-Aldrich Corp.). The RT-quantitative PCR was run and validated as described previously (Ndiaye et al., 2008) for a maximum of 35 cycles, under the following conditions: denaturing at 94 °C for 30 s, annealing at 60 °C for 60 s and extension at 72°C for 60 s. Concentrations of NPY, AgRP and POMC were normalized to peptidylprolyl isomerase B (cyclophilin B) mRNA expression in the same sample to determine the relative mRNA concentrations of NPY, AgRP and POMC. The homologous standard curve prepared from purified NPY, AgRP and POMC cDNA PCR product was used to calculate the steady-state concentration of NPY, AgRP and POMC mRNA in triplicate wells for each sample. The PCR amplification products were electrophoretically separated on 1.5% agarose gels and visualized with ethidium bromide. For initial validation, the specific band corresponding to the size of the expected NPY, AgRP and POMC cDNA fragment was cut and purified using the QIAquick Gel Extraction kit (Qiagen Sciences) for sequence confirmation. A control sample that was not reverse transcribed was used to confirm that the product obtained was not amplified from genomic DNA.

The data were analysed as an incomplete block design using MIXED model procedures of SAS (Version 9.1; SAS Institute, Cary, NC, USA) testing the random effects of lamb and block, and the fixed effects of treatment. Because block effect was not significant ($p > 0.10$) for any of the three variables, the block effect was removed from the model. Because the objective of the experiment was to evaluate the effect of the metabolites and hormones on mRNA concentration, the control treatment (low glucose) was compared with the individual treatments using Dunnett's mean separation procedures.

Results and discussion

Several neural cell lines expressing neuropeptides that regulate food intake have been successfully used to test direct effect of nutrients and hormones on the regulation of gene expression (Lee et al., 2005; Cai et al., 2007). However, these *in vitro* models are unable to completely represent *in vivo* conditions because studies using neuronal cell lines cannot integrate interactive functions of various cell types in the hypothalamus in response to complex signals from the whole body. Considering these limitations, we chose ex vivo cultures of sheep hypothalamus slices in the present studies to investigate the regulation of expression of neuropeptide genes in response to hormones and nutrients.

The hypothalamic concentration of NPY and AgRP mRNA did not change because of HG or glucose and insulin incubation treatments ($p = 0.18$ and

$p > 0.20$; Figs 1 and 2, respectively). However, the relative concentration of POMC mRNA was increased by the combination of insulin and glucose ($p < 0.01$; Fig. 3), but was not affected by HG alone. Lee et al. (2005) showed a decrease in NPY and AgRP mRNA concentration in mice hypothalami incubated with increasing concentrations of glucose, but reported no changes in POMC concentrations in response to glucose. These effects of glucose on NPY and AgRP expression in mice (Lee et al., 2005) support the glucostatic theory of regulation of feed intake proposed by Mayer (1953), and the lack of an effect of glucose on these neuropeptides in our study supports the hypothesis that glucose has a minimal role as a regulator of appetite in ruminants (Manning et al., 1959). However, in the present experiment, there was an effect of HG on POMC expression in the presence of insulin, suggesting that under certain conditions glucose may have an effect on specific neuropeptides in ruminants, but that insulin is required to mediate that effect. Previous studies have shown that insulin potentiates or enables central effects of numerous metabolites and hormones on voluntary intake, including glucose and CCK (for reviews see: Forbes, 1988; Schwartz et al., 1992a,b).

Diabetic animals with high blood glucose and low insulin levels commonly have hyperphagia (Booth, 1972). These animals also have an increased expression of the orexigenic peptides (NPY and AgRP) and a reduced expression of anorexigenic POMC in the hypothalamic arcuate nucleus (Williams et al., 1998; Sindelar et al., 2002). This indicates that without insulin action, glucose alone cannot effectively induce a satiety signal in the hypothalamus of diabetic animals. In this regard, our *ex vivo* results showing that neither insulin nor glucose alone had an effect on POMC expression, but the combination of glucose and insulin increased POMC expression. This suggests that as observed in non-ruminants, insulin is required for effects of

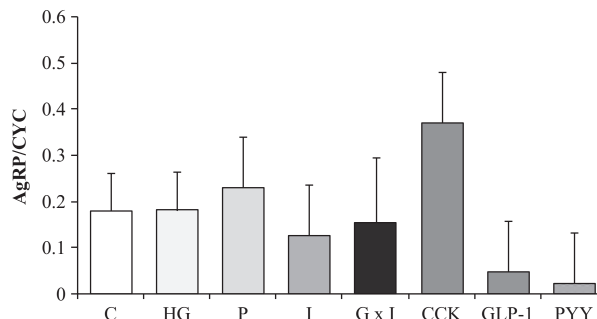

Fig. 2 *In vitro* hypothalamic sheep aguti-related peptide (AgRP) mRNA concentration (normalized with cyclophilin B; CYC) after 2 h incubation in DMEM containing the treatments: control (C; 1 mM of glucose), high glucose (10 mM of glucose), propionate (P; 1 mM of Na propionate and 1 mM of glucose), insulin (I; 1 nM of bovine insulin and 1 mM glucose), glucose–insulin (G + I; 1 nM of insulin and 10 mM of glucose), cholecystokinin (CCK; 80 pM of CCK-8 sulphide and 1 mM glucose), glucagon-like peptide-1 (GLP-1; 120 pM of GLP-1 (7-36) amide and 1 mM of glucose) and peptide YY (PYY; 100 pM of bovine PYY_{3-36} and 1 mM of glucose).

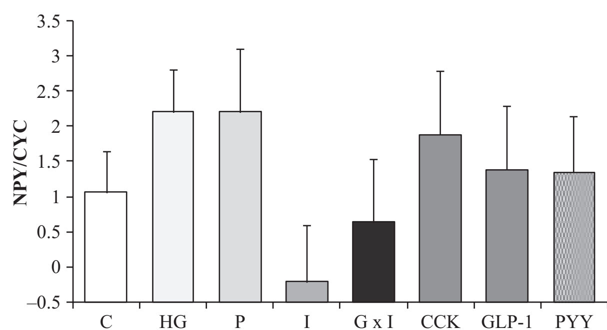

Fig. 1 *In vitro* hypothalamic sheep neuropeptide Y mRNA concentration (normalized with cyclophilin B; CYC) after 2 h incubation in DMEM containing the treatments: control (C; 1 mM of glucose), high glucose (10 mM of glucose), propionate (P; 1 mM of Na propionate and 1 mM of glucose), insulin (I; 1 nM of bovine insulin and 1 mM glucose), glucose–insulin (G + I; 1 nM of insulin and 10 mM of glucose), cholecystokinin (CCK; 80 pM of CCK-8 sulphide and 1 mM glucose), glucagon-like peptide-1 (GLP-1; 120 pM of GLP-1 (7-36) amide and 1 mM of glucose) and peptide YY (PYY; 100 pM of bovine PYY_{3-36} and 1 mM of glucose).

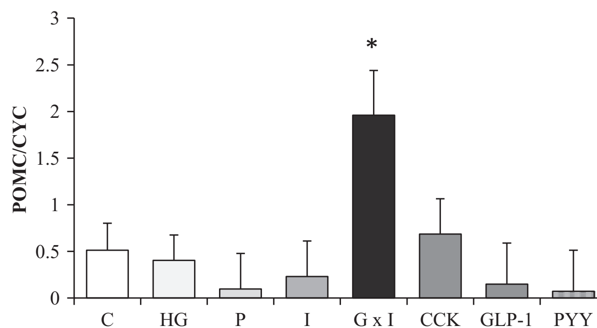

Fig. 3 *In vitro* hypothalamic sheep proopiomelanocortin mRNA concentration (normalized with cyclophilin B; CYC) after 2 h incubation in DMEM containing the treatments: control (C; 1 mM of glucose), high glucose (10 mM of glucose), propionate (P; 1 mM of Na propionate and 1 mM of glucose), insulin (I; 1 nM of bovine insulin and 1 mM glucose), glucose–insulin (G + I; 1 nM of insulin and 10 mM of glucose), cholecystokinin (CCK; 80 pM of CCK-8 sulphide and 1 mM glucose), glucagon-like peptide-1 (GLP-1; 120 pM of GLP-1 (7-36) amide and 1 mM of glucose) and peptide YY (PYY; 100 pM of bovine PYY_{3-36} and 1 mM of glucose). *$p < 0.01$ for G + I compared with C.

elevated glucose concentration on hypothalamic expression of mRNA for POMC in ruminants. Given the action of insulin in enhancing glucose uptake, insulin likely facilitated glucose uptake by the cells of sheep hypothalamus and generated metabolic signals to enhance anorexigenic POMC expression. In addition, insulin alone did not affect POMC expression, further suggesting hypothalamic glucose is a mediator of insulin-induced satiety signals.

In the present study, there was no effect of propionate on concentrations of mRNA for NPY, AgRP or POMC ($p \geq 0.18$); thus, it seems likely that direct effects on the hypothalamus is not the primary mechanism by which propionate effects DMI in ruminants. As mentioned previously, the concentrations of propionate used were estimated to be three times the concentrations reported for jugular vein or arterial blood of ruminants. In lactating dairy cows, a decrease in DMI because of ruminal propionate infusion (Oba and Allen, 2003) was associated with an increase in plasma concentration of glucose, insulin and propionate. The effect of propionate on DMI has been attributed to an increase in propionate oxidation in the liver (Anil and Forbes, 1988; Allen et al., 2009).

There was no effect of insulin ($p \geq 0.18$) on mRNA for the hypothalamic neuropeptides measured when insulin was added to the incubation media in combination with a low concentration of glucose. Sato et al. (2005) also reported no change in NPY mRNA concentration in slices of rat hypothalamus incubated with increased concentrations of insulin *in vitro*. However, a decrease in mRNA NPY concentration was observed in fasted rats that received intracerebroventricular (ICV) infusions of insulin. Similar to the results reported in the present study, Benoit et al. (2002) reported an increase in POMC mRNA concentration in fasted rats receiving ICV insulin infusions every 12 h. Benoit et al. (2002) also reported a decrease in feed intake when rats received an ICV insulin infusion 1 h before feeding. The results of the present study, where effects of insulin on POMC mRNA concentration were only observed in the presence of a HG concentration, suggest that the effects of insulin may require glucose. Grovum (1995) has previously suggested that insulin decreases DMI when plasma glucose concentration is elevated above a basal concentration. In this regard, reductions in DMI have been reported in lactating dairy cows during insulin infusions for which euglycemia is maintained through intravenous glucose infusions (Leury et al., 2003). Even though an increase in POMC mRNA concentration was observed in the present study, the effect of POMC on DMI in ruminants has not been reported.

Intracerebroventricular infusion of CCK decreases DMI and meal size in sheep (Della-Fera and Baile, 1980), and similar results have been observed in non-ruminants (Bi et al., 2004). However, Bi et al. (2004) showed that the changes in NPY expression because of CCK occurred in rats, but not mice, suggesting differences in the effects of CCK on the hypothalamus across species. In the present study, there were no effects of CCK on NPY, AgRP or POMC mRNA concentration ($p \geq 0.18$). However, as for glucose, the effect of CCK on appetite and neuropeptide expression in the hypothalamus may be insulin dependent (Schwartz et al., 1992a,b).

In vitro hypothalamic incubation in media containing GLP-1 did not change mRNA concentration for NPY, AgRP or POMC ($p \geq 0.18$). This result is similar to previous *in vivo* results in rats (Turton et al., 1996), where ICV infusion of GLP-1 did not change mRNA concentration for NPY compared with saline-infused rats. However, Seo et al. (2008) showed that ICV infusion of GLP-1 decreased NPY and AgRP and increased POMC mRNA concentration in the hypothalamus in 48-h fasted rats. These changes in mRNA concentration for the different neuropeptides were associated with changes in feed intake. Based on the results of the current study and the discrepancy in the results reported for non-ruminants, the effect of GLP-1 on the hypothalamic neuropeptides that regulate intake cannot be confirmed.

Peptide YY decreases DMI in non-ruminants (Batterham et al., 2002), and in rats, the central effect of PYY is by binding to a Y 2 receptor, which illicits a decrease in NPY gene expression (Batterham et al., 2002; Challis et al., 2003). In mice, PYY also increases mRNA concentration for POMC (Challis et al., 2003). In the present study, no changes were observed in mRNA concentration for NPY, AgRP and POMC ($p \geq 0.18$) when bovine PYY was added to the media. The effects of PYY on DMI have not been reported for ruminants. Onaga et al. (2000) reported in sheep that plasma PYY concentration did not change over a 2-day period, even after the ingestion of diets based on either forage or concentrate. Therefore, it is possible that PYY does not play a role in the regulation of DMI in ruminants. However; it is also possible that the functional structure of bovine PYY is not similar to ovine PYY. A different structure of the peptide could change the three-dimensional conformation of the peptide, which would affect the binding of the peptide to the receptor (Keire et al., 2000). Finally, it is possible that the bovine PYY

synthesized for use in the present study did not have the same conformational structure, and thus functionality, as the endogenous peptide.

In conclusion, the incubation of sheep hypothalami in media containing metabolites or hormones by themselves did not change mRNA concentration for the neuropeptides NPY, AgRP or POMC. However, the combination of insulin and glucose increased POMC mRNA concentration, suggesting a role for insulin in regulating intake in ruminants that requires glucose, or vice versa. While POMC has demonstrated effects on intake in non-ruminants, the direct role of hypothalamic POMC in the regulation of food intake in ruminants requires further investigation.

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